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Modified Radioimmunoassay Determination for Staphylococcal Enterotoxin B in Foods

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The sensitivity of solid-phase radioimmunoassay for the measurement of staphylococcal enterotoxin B (SEB) in foods was decreased by food constituents that react with rabbit anti-SEB with an equivalency of over 2 ng/ml. This activity was minimized by a conditioning step for anti-SEB and by removal of interfering compounds in the sample by extraction. The assay was a sequential solid-phase radioimmunoassay technique in which polystyrene test tubes were initially incubated with antisera and then with bovine serum albumin. The tubes were then conditioned with either a centrifuged aqueous cheese extract or, equally effective, reconstituted nonfat dry milk for 16 h at 4°C. Samples of milk or heat-treated and $CHCl_3$-extracted cheese or chicken salad slurries were incubated in the assay tubes for 6 h at 37°C. The samples were replaced by ^{125}I-labeled SEB and incubated for a further 2 to 4 h before the contents were removed and the tubes were washed and counted. A buffer solution containing known concentrations of toxin served as standards for assaying SEB in the food extracts. The entire assay can be accomplished within 24 h with a sensitivity of 1 ng/ml in milk and in the cheese extract or 1.3 ng/ml in the chicken salad extract.

Solid-phase radioimmunoassay techniques capable of detecting small quantities of staphylococcal enterotoxins in foods have recently been developed. Collins et al. (5, 6) published a procedure for assaying staphylococcal enterotoxins A (SEA) and B (SEB) employing bromacetylcellulose particles capable of quantitating 20 ng of toxin per g of food. Johnson et al. (9, 10) adapted polystyrene tubes as the solid phase and, using food extracts, indicated a potential recovery of 2 to 3 ng/g of SEA and SEB in food, with no significant cross-reactivity. However, they failed to provide statistical evaluation to determine the actual sensitivity and precision of their assay procedure. Moreover, it would be advantageous to be able to use one buffer for the standard curve to determine the concentration of toxin for a variety of food items whose history is unknown. The alternative is to employ a separate standard curve for each food item obtained from a comparable, toxin-free, food source. In attempting to assay cheese slurries for SEB in the present study, it was found that interference from food constituents was sufficiently large to seriously reduce the sensitivity and precision of the assay.

A procedure is described which minimizes the nonspecific reactivity in the anti-SEB preparations, by use of conditioned tubes, and the interference by food components (FC) by an extraction procedure utilizing SEB standards in buffer for assaying for SEB in milk, cheese, and chicken salad.

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MATERIALS AND METHODS

Reagents. All reagents were prepared in a buffer consisting of phosphate-buffered saline (PBS; 0.07 M NaCl, 0.07 M phosphate, and 0.1% sodium azide) adjusted to pH 7.2 with 5 N NaOH and supplemented with 1% bovine serum albumin (PBS-BSA; Sigma Co.). Where indicated, the PBS was used without BSA supplementation.

The antisera preparations (Makor Chemicals, Ltd., Jerusalem, Israel) were supplied in vials containing approximately 2.8 mg of antibody as a lyophilized, sodium sulfate-precipitated fraction of rabbit serum (personal communication). Each vial was reconstituted with 2 ml of PBS before use.

In a comparison study, three additional lots of Makor antisera, as well as antisera obtained from M. S. Bergdoll, Food Research Institute, Madison, Wis., and J. F. Metzger and S. J. Silverman, Ft. Detrick, Md., were evaluated. Homologous purified SEB was also supplied by M. S. Bergdoll. The toxin was diluted in PBS-BSA buffer for use in standards and for assay experiments with food samples.

Preparation of assay tubes. The antibody-coated polystyrene assay tubes were prepared by the procedure described by Johnson et al. (9). Antisera was diluted 1:1,000 before use, a concentration sufficient

for saturating all of the available antibody sites in the assay tubes.

The antibody-coated assay tubes were conditioned either by incubation overnight at 4°C with 2 ml of reconstituted nonfat powdered milk (Carnation Co., 20 g/100 ml of PBS, adjusted to pH 7.2 with 5 N NaOH) or with an aqueous cheese extract (AE), whose preparation is described below. The tubes were then emptied, washed with 2 ml of PBS, and stored at 4°C. A simple suction apparatus with a Pasteur pipette was used for removal of solutions from assay tubes. The conditioned and antibody-coated assay tubes were stable for at least 7 days.

Iodination. The iodination of SEB was based upon the chloramine T procedure described by Greenwood and Hunter (8) and modified by Johnson et al. (10) and Kauffman and Johnson (11) for staphylococcal enterotoxins. The specific activity of the ^{125}I-labeled SEB used in this study was approximately 40 μCi/μg of protein. The freshly iodinated antigen was titrated by adding 1.0-ml aliquots of different concentrations of the labeled SEB to the assay tubes. After incubation at 37°C with shaking (laboratory rotator model G-2, New Brunswick Scientific Co.) at 200 rpm for 2 h, the tubes were emptied, washed with 2 ml of PBS, and counted for 1 min. The linear plot of the amount of labeled SEB added to each tube against the percentage of that SEB bound to the antibody on the assay tube resulted in a hyperbola. The amount to be added to each assay tube for assaying SEB corresponded to the point on the hyperbola derived by bisecting the angle formed by the intersection of the two asymptotes.

Radioimmunoassay. In the procedure for the assay of staphylococcal enterotoxin in food, 1.0 ml of either a standard or a food extract (see below) was added to each tube. The assay tubes were incubated for 4 h at 37°C with shaking (laboratory rotator model G-2, New Brunswick Scientific Co.). Samples could be incubated as long as 18 h without an appreciable change in sensitivity or precision. The samples or standards were removed, and the tubes were washed with 2 ml of PBS. A 1.0-ml quantity of the standardized ^{125}I-labeled SEB was then added to each tube. The tubes were then shaken for 2 h at 37°C, the contents were emptied, and the tubes were washed with 2 ml of PBS and then counted.

Preparation of food samples. (i) Whole milk. Commercial pasteurized whole milk was adjusted to pH 7.2 with 5 N NaOH, and sodium azide (0.2 g/100 ml) was added as a preservative.

(ii) Powdered milk. Ten grams of instant nonfat dry milk (Carnation Co.) was reconstituted with 100 ml of PBS-BSA buffer, and the pH was adjusted to 7.2 with 5 N NaOH.

(iii) Cheddar cheese. Aged cheddar cheese, purchased locally, was prepared either as an AE by the method of Johnson et al. or, after further fractionation, by heat and a chloroform extraction (HCE). The procedure for preparing the HCE was based upon those described by Read et al. (13) and Reiser et al. (14).

The AE was prepared by blending (Tekmar Co., model SDT) 10 g of commercial cheddar cheese with 20 ml of PBS in a 35-ml round-bottom centrifuge tube for 3 min and adjusting the slurry to pH 7.2 with 5 N NaOH. In later experiments, a more concentrated preparation consisting of 10 g of cheese in 10 ml of PBS proved to be equally suitable for assaying. The solids were removed by centrifugation (Sorvall RC2B, rotor SS34) at 18,000 rpm for 20 min at 4°C. All subsequent centrifugations were also performed at this speed and temperature, and for the same period of time. The supernatant was removed and, where indicated, used for conditioning assay tubes.

The HCE sample was prepared by further adjusting the AE to pH 4 to 4.5 with 2 N HCl. The resulting suspension was then maintained in a water bath at 50°C for 30 min, after which it was centrifuged. The pH of the supernatant was adjusted to 7.2 with 5 N NaOH, and the resultant precipitate was removed by centrifugation. The supernatant was then extracted with chloroform (1:1), the extraction mixture was centrifuged, and the aqueous phase was used for the assay of SEB.

Known concentrations of SEB were usually added to the HCE, because recovery experiments demonstrated that the heat treatment and extraction procedure had no statistically significant effect on quantitation.

(iv) Chicken salad. Chicken salad was obtained from a local commercial source. A 10-g sample of chicken salad was blended with 10 ml of PBS-BSA buffer for 3 min, and the pH was adjusted to 7.2 with 5 N NaOH. The aqueous extract was incubated at 50°C for 30 min, the solids were removed by centrifugation, the soluble portion was extracted with chloroform (1:1) and centrifuged, and the aqueous phase was assayed for toxin.

Counting equipment. ^{125}I was measured in a gamma counter, (Baird Atomic, model 707), with a counting efficiency of approximately 50% and a background count of approximately 30 cpm.

Statistical analysis. Assay values of known concentrations of SEB in food and in buffer were analyzed by computing (UNIVAC 1106 computer) the line of best fit as derived from third-order curves by polynomial regression. The 95% confidence limits were also determined. Unless otherwise indicated, samples were prepared in duplicate, and each of the duplicates was assayed in triplicate. Samples were further compared by analysis of variance, and the significance of the differences was determined by the t test.

RESULTS

A comparison of the assay for SEB in buffer and reconstituted nonfat powdered milk is shown in Fig. 1. The 95% confidence limits were omitted from the figure for the sake of clarity. There were significant differences ($P > 0.05$) between values obtained for the same concentrations of SEB in unconditioned tubes in buffer and in milk, invalidating any comparison. The curves obtained for SEB in buffer and in milk, using conditioned tubes, were in agreement (P

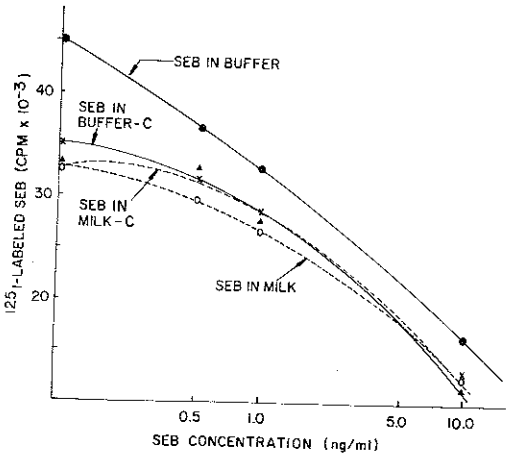

FIG. 1. *Comparison of assay for SEB in PBS-BSA buffer and reconstituted nonfat powdered milk, with conditioning (C) and without conditioning of the assay tubes. The points were from single samples assayed in triplicate, with the best-fit line calculated by polynomial regression.*

< 0.05) with those values obtained for milk in unconditioned tubes.

The data in Fig. 1 are recalculated and presented in Table 1 to show the assay values obtained when, for conditioned and unconditioned tubes, buffer standards are used. The standard deviation of unconditioned tubes is larger than for conditioned tubes. The values of 1 ng/ml and below are statistically similar, and the assay values recovered are too high. In contrast, with conditioned tubes, one could distinguish 1 ng/ml in milk and, as stated above for Fig. 1, the assay values are identical to the values obtained from buffer standards ($P <$ 0.05).

A similar sensitivity in detecting SEB is obtained when assaying whole milk (Fig. 2). Using conditioned tubes, quantitative recovery is obtained for concentrations of 0.5, 1.0 and 5.0 ng of SEB per ml.

The incubation of an AE of cheddar cheese (without added SEB) in unconditioned assay tubes interferes with the uptake of labeled toxin (Table 2). The interference, as demonstrated by the reduction of ^{125}I-labeled toxin uptake, ranges from 18 to over 50%. The mean reduction was 34.62% and the high standard deviation of 12.18% indicated a high variability inherent in the use of this type of preparation. The inhibition in binding corresponded to an uptake of labeled iodine representing a mean of 2.32 ng of toxin per ml in the cheese samples, with an equivalent magnitude in standard deviation.

The effectiveness and need for the HCE procedure, in addition to the advantage of employing conditioned assay tubes, are illustrated in Table 3. The values obtained when assaying an AE in unconditioned tubes were excessively high, as was the standard deviation. The HCE procedure decreased the standard deviation, and the assay values were more in agreement with the buffer standards, but there still re-

TABLE 1. *Comparison of unconditioned and conditioned assay tubes in the determination of SEB in reconstituted nonfat powdered milk*

Concn[a] (ng/ml)	Unconditioned tubes[a] (ng/ml)	Conditioned tubes[a] (ng/ml)
0	1.19 ± 0.30[c]	0.32 ± 0.10[f]
0.5	1.77 ± 0.34[c]	0.87 ± 0.46[f,g]
1.0	2.82 ± 0.65[c]	1.27 ± 0.21[g]
5.0	14.27[b] ± 1.10[d]	6.70[e] ± 0.46[h]
10.0	22.25 ± 7.31[e]	12.75 ± 1.25[i]

[a] Values for each treatment were obtained by comparison to their respective standard curves in PBS-BSA buffer as indicated in the first column.

[b] Results obtained from a single sample in triplicate ± standard deviation. All the others represent duplicate samples assayed in triplicate ± standard deviation.

[c-i] Values having the same letter are not statistically different ($P < 0.05$).

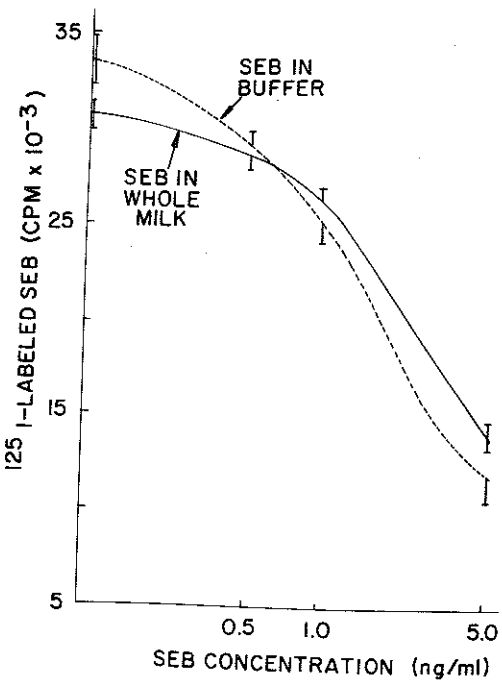

FIG. 2. *Comparison of assay for SEB in PSB-BSA buffer and in whole milk using conditioned assay tubes. The points were from single samples assayed in triplicate, with the best-fit line calculated by polynomial regression.*

mained an inability to differentiate the samples containing a zero concentration from those containing 1 ng of SEB per ml. The use of conditioned tubes coupled with the HCE treatment reduced the standard deviation to acceptable levels, and the actual assay values were in closer agreement with the predicted concentrations of SEB in buffer, resulting in an ability to quantitate 1 ng of SEB per ml. Reconstituted powdered skim milk was as effective as an AE for conditioning assay tubes.

The use of conditioned tubes and a HCE procedure also improved the ability to assay SEB in chicken salad (Table 4); however, the improvement in sensitivity and in the standard deviations were less than that noted for cheese and milk. Although differences between the values obtained for the samples containing 1.0 ng of toxin per ml could not be differentiated from the samples without added toxin, differences between the 1.0-, 5.0-, and 10.0-ng/ml concentrations were statistically significant ($P > 0.05$). Analysis of this data, using the 95% confidence limits of the regression line to calculate the mean concentration of SEB that would be statistically detectable, indicates that a concentration of 1.3 ng/ml is necessary.

TABLE 2. *Effect of incubation of assay tubes with AE[a] on the uptake of ^{125}I-labeled SEB*

Expt[b]	Reduction in ^{125}I uptake	
	%	Equivalent SEB (ng/ml)
1	18.2	0.57
2	33.7	2.82
3	25.2	1.63
4	18.0	0.91
5	43.7	ND[c]
6	37.2	0.95
7	38.4	2.13
8	45.4	7.95
9	27.8	1.60
10	58.6	ND
Mean ± SD	34.62 ± 12.81	2.32 ± 2.39

[a] Without added SEB. See text for preparation of AE.
[b] Each experiment was done in triplicate on different sources of cheese.
[c] ND, Not done.

DISCUSSION

Bergdoll et al. (3) consider an emetic dose for humans as being less than 1,000 ng of SEB and that, consequently, an assay technique should be capable of detecting at least 2 ng/g of food. The detection of this concentration of SEB is within the inherent capabilities of the radioimmunoassay technique, and this fact can readily be demonstrated with buffer standards. The difficulty in achieving this sensitivity in foods appears to be due to interference by FC in nonspecific reactions with the absorbed antibodies in the assay tube.

The presence of FC has been noted by several investigators, with indications that the responsible factors are largely proteins (3, 7, 9); however, one cannot exclude the possibility that other constituents, such as lipids, are involved. With unconditioned assay tubes, the presence of FC is equivalent to 1.19 ± 0.30 ng/ml in milk and 2.47 ± 0.79 ng/ml in cheese slurry. When milk is assayed in conditioned assay tubes, the result is statistically identical with that from the buffer standards, but a cheese slurry still has an appreciable concentration of FC components. It is therefore necessary to extract both cheese and chicken salad samples to make

TABLE 3. *Comparison of recovery of SEB from cheese samples using unconditioned assay tubes, or tubes conditioned with cheese slurry or with reconstituted nonfat powdered milk*

Concn[a] (ng/ml)	Recovery of SEB[b] (ng/ml) in assay tubes			
	Unconditioned		Conditioned with:	
			Cheese slurry	Milk
	SEB in slurry	SEB in extract	SEB in extract	SEB in extract
0	2.47 ± 0.79[d]	1.44 ± 0.42[d]	0.49 ± 0.20[g]	0.57 ± 0.35[g]
0.5	ND[c]	ND	0.72 ± 0.19[g]	0.55 ± 0.19[g]
1.0	14.07 − 14.79[e]	2.52 − 1.03[d]	1.14 ± 0.49[h]	1.15 ± 0.19[h]
5.0	ND	ND	6.04 ± 1.40[i]	4.91 ± 0.89[i]
10.0	43.67 ± 22.55[f]	13.55 ± 8.59[e]	12.25 ± 1.17	11.83 ± 2.04[e]

[a] Concentration of SEB in PBS-BSA buffer standards.
[b] Values for each treatment were obtained by comparison to their respective standard curves in PBS-BSA buffer.
[c] ND, Not done.
[d–i] Values having the same letter are not statistically different ($P < 0.05$).

TABLE 4. *Comparison of the assay for SEB in chicken salad slurry and extract using conditioned and unconditioned assay tubes*

Concn[a] (ng/ml)	Recovery of SEB (ng/ml)[b] in assay tubes:			
	Unconditioned		Conditioned[c]	
	chicken salad slurry[d]	chicken salad extract[e]	Chicken salad slurry	Chicken salad extract
0	2.03 ± 0.23[g]	1.09 ± 0.57[h]	1.67 ± 0.55[k]	0.72 ± 0.16[m]
0.5	ND[f]	1.10 ± 0.41[h]	ND	0.64 ± 0.15[m]
1.0	2.20 ± 0.44[g]	1.46 ± 0.94[h]	3.53 ± 0.25[l]	0.87 ± 0.33[m]
5.0	ND	3.50 ± 1.55[i]	ND	3.60 ± 0.84[n]
10.0	ND	6.35 ± 1.30	ND	8.03 ± 2.98[o]

[a] Concentration of SEB in PBS-BSA buffer standards.
[b] Values were obtained by comparison of samples to their respective standard curves in PBS-BSA buffer.
[c] Conditioned with nonfat powdered milk for 16 h at 4°C.
[d] Single sample in triplicate ± standard deviation.
[e] Duplicate samples in triplicate ± standard deviation.
[f] ND, Not done.
[g–o] Values having the same letter are not statistically different ($P < 0.05$).

them more comparable to the buffer standards. The use of PBS-BSA buffer standards obviates any need to use a toxin-free food source for standards. Incorporating the use of conditioned assay tubes and an extraction procedure, one can achieve a sensitivity of 1 ng/ml in milk or a cheese extract and 1.3 ng/ml in a chicken salad extract. This is equivalent to the detection of 2 to 2.6 ng/g in the original cheese or chicken salad. To detect levels of toxin below 2 ng, a modest twofold concentration of the sample would be sufficient. Higher concentrations might cause progressively troublesome interference by FC unless they are selectively eliminated. If less sensitivity is acceptable, greater than 5 ng/ml of extract, accompanied by less precision, then the use of conditioned tubes may not be necessary, but an extraction step is still required. The radioimmunoassay procedure suggested in this study can be completed within a 24-h period. Employing a competitive radioimmunoassay procedure, recently suggested as being of equal sensitivity (12), could shorten the procedure considerably. One should be cautioned that the techniques described in this publication for milk, cheese, and chicken salad may not be as effective for other types of food.

The sensitivity attained in this study may be limited by the quality of the reagents available. The antisera used for this study, source 1-A in Table 5, was the most potent commercially available lot tested. The relative binding capacity of lots from the same manufacturer and from other sources varied from 0.18 to 2.19, but the antiserum with the highest binding capacity, from a noncommercial source, is no longer available. Unlike Askenase and Leonard (1), we did not find anomalous behavior when the polystyrene tube method was used with whole antisera. Antisera having relative binding capacitities above 0.3, independent of whether they were whole antisera or the sulfate-precipitated fraction, were sufficiently active for use in the assay. Since that level of activity could be obtained with the homologous antisera, purification by sulfate precipitation may not be necessary. The SEB antigen is also heterogeneous, and the different forms are not distinguished by serological techniques. Chesbro et al. (4) have recently indicated that ratios of the various forms of SEB change during the growth

TABLE 5. *Maximum amount of labeled antigen complexed by a unit of antisera*

Source[a]	Protein concn (μg/ml)	Relative maximum binding capacity[b]
1-A	45	1.00
1-B	50	0.34
1-C	20	0.17
1-D	20	0.35
2		0.40
3		0.18
4		2.19

[a] Sources 1, 2, and 3 were rabbit antisera; 4 was burro antisera. Source 1 was purified by precipitation with sulfate, and 2, 3, and 4 were homologous antisera.

[b] A 1.0-ml volume of each of several antisera was added to polystyrene tubes. The concentration was sufficient to bind all available sites on the polystyrene. The antibody was removed after 2 h and replaced by 1 ml of ^{125}I-labeled SEB and incubated for an additional 2 h. The tubes were washed and counted. The relative binding capacity is the ratio of each antisera to source 1-A.

phases and that, although the antiserum detects a number of these forms, the antiserum usually available is derived from the antigen mixture produced during the late logarithmic-stationary phases. The relative affinity of these homologous reagents has not been ascertained.

LITERATURE CITED

1. **Askenase, P. W., and J. Leonard.** 1970. Solid phase radioimmunoassay of human B1C globulin. Immunochemistry 7:29–41.
2. **Bergdoll, M. S.** 1973. Enterotoxin detection, p. 287–292. *In* B. Hobbs and J. H. B. Christian (ed.), The microbiological safety of food. Academic Press Inc., London.
3. **Bergdoll, M. S., R. Reiser, and J. Spitz.** 1976. Staphylococcal enterotoxins—detection in food. Food Technol. 30:80–84.
4. **Chesbro, W., D. Carpenter, and G. J. Silverman.** 1976. Heterogeneity of *Staphylococcus aureus* enterotoxin B as a function of growth stage: implications for surveillance of foods. Appl. Environ. Microbiol. 31:581–589.
5. **Collins, W. S., J. F. Metzger, and A. D. Johnson.** 1972. A rapid solid phase radioimmunoassay for staphylococcal B enterotoxin. J. Immunol. 108:852–856.
6. **Collins, W. S., A. D. Johnson, J. F. Metzger, and R. W. Bennett** 1973. Rapid solid-phase radioimmunoassay for staphylococcal enterotoxin A. Appl. Microbiol. 25:774–777.
7. **Genigeogis, C., and I. K. Kuo.** 1976. Recovery of staphylococcal enterotoxin from foods by affinity chromatography. Appl. Microbiol. 31:274–279.
8. **Greenwood, F. C., and W. M. Hunter.** 1963. The preparation of ^{131}I-labeled human growth hormone of high specific radioactivity. Biochem. J. 89:114–123.
9. **Johnson, H. M., J. A. Bukovic, and P. E. Kauffmann.** 1973. Staphylococcal enterotoxins A and B: solid-phase radioimmunoassay in food. Appl. Microbiol. 26:309–313.
10. **Johnson, H. M., J. A. Bukovic, P. E. Kauffman, and J. T. Peeler.** 1971. Staphylococcal enterotoxin B: solid-phase radioimmunoassay. Appl. Microbiol. 22:837–841.
11. **Kauffman, P. E., and H. M. Johnson.** 1975. Stability of ^{125}I-labeled staphylococcal enterotoxins in solid-phase radioimmunoassay. Appl. Microbiol. 29:776–779.
12. **Pratt, J. J.** 1975. Disadvantages of sequential saturation method. Clin Chem. 21:2002–2004.
13. **Read, R. B., Jr., J. Bradshaw, W. L. Pritchard, and L. A. Black.** 1965. Assay of staphylococcal enterotoxin from cheese. J. Dairy Sci. 48:420–424.
14. **Reiser, R., D. Conaway, M. S. Bergdoll.** 1974. Detection of staphylococcal enterotoxin in foods. Appl. Microbiol. 27:83–85.